12. Crystallization of the Cytochrome bc_1 complex

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A significant fraction of the proteins in any genome are membrane proteins, and membrane proteins are vital to many fundamental processes of life. While many membrane proteins have now been crystallized in three dimensions, membrane proteins have proven considerably more difficult to crystallize than soluble proteins, and some have not yielded after extensive efforts at crystallization, e.g., the mitochondrial adenine nucleotide transporter, the bacterial chemotaxis receptor, and lactose permease.

One membrane protein that crystallizes fairly readily is the mitochondrial cytochrome ***bc*$_1$** complex. This 11-subunit respiratory enzyme has been crystallized in at least eight different crystal forms. We here review conditions that lead to crystallization in various forms, and provide some of the history of our crystallization attempts.

12.1. About the Protein

The cytochrome ***bc*$_1$** complex is a redox enzyme, E.C.1.10.2.2, which oxidizes ubiquinol and reduces cytochrome ***c***. The cytochrome ***b*$_6$*f*** complex is a partly homologous complex in chloroplasts or cyanobacteria that oxidizes plastoquinol and reduces plastocyanin or a c-type cytochrome. We refer to the superfamily that includes these and related redox proteins[1] as ***bc*** complexes. Mitochondrial ***bc*$_1$** complexes contain 10-11 subunits in the monomer, while the bacterial ***bc*$_1$** complexes thus far characterized have 3 to 4. This chapter will cover only the mitochondrial ***bc*$_1$** complexes, although crystals have been obtained with bacterial ***bc*$_1$**[2] and ***b*$_6$*f***[3] complexes. Fig. 12.1 shows the overall structure of the mitochondrial complex. It has considerable hydrophilic protein extending into the aqueous phases on either side of the membrane, making it a good candidate for forming membrane protein crystals of type II as defined by Michel.[4]

12.2. Purification Procedures

There are four principal purification methods, and crystals have been obtained from protein purified by at least three of them. The first two methods, which we will refer to as

the Rieske and Yu methods, rely mainly on salt fractionation in bile salt detergents to separate the ***bc*$_1$** complex from other proteins of the mitochondrial inner membrane.

The Rieske method(s) developed in collaboration with Hatefi and other co-workers at the Enzyme Institute in Madison, uses a "red-green split"[5] in DOC/KCl to separate oxidase (in the pellet of insoluble material after centrifugation) from other respiratory complexes, which are solubilized and remain in the supernatant. Then, one of several fractionation schemes involving DOC/NH_4OAc and/or cholate/$(NH_4)_2SO_4$,[6-8] is used to separate the dehydrogenases and other contaminants from the ***bc*$_1$** complex.

The Yu method[9] prepares "***bc*$_1$** complex III" from pure complex II+III (succinate:cytochrome ***c*** reductase, or SQR). SQR is extracted at pH 10.5, which solubilizes a 2-subunit succinate dehydrogenase. The pellet is fractionated in DOC with $(NH_4)_2SO_4$[9] or NH_4OAc[10] to remove the small subunits of complex II.

The chromatographic purification of Schagger, von Jagow, and co-workers uses hydroxyapatite chromatography starting with a Triton X-100 detergent extract of mitochondria, with[11] or without[12] antimycin. An updated description of this method has appeared.[13] Musatov and Robinson[14] developed a procedure that combines the selective Triton extraction of the Schagger method with the Rieske salt fractionation in bile salts.

The authors of this chapter use a chromatographic procedure involving anion exchange chromatography of lauryl maltoside extracts. This procedure has been applied to isolation of cytochrome ***bc*$_1$** complexes from α-proteobacteria,[15-21] firmicutes,[22,23] and from mitochondria of animals,[17] plants,[24] fungi,[17] *Chlorophyta,*[25] and trypanosomes.[26] The power of this method lies in the fact that ***bc*$_1$** complexes from all these organisms are very tightly adsorbed to anion exchange columns, even at ionic strength 340 mM (260 mM NaCl, 50 mM KPi pH 7.5) which elutes the vast majority of mitochondrial proteins. The material eluted in the ***bc*$_1$** peak in a gradient from 260 to 500 mM NaCl in 50 mM KPi is already highly purified.

Column-binding affinity is not expected to be highly conserved phylogenetically. The generality of this procedure is probably due to the fact that the cytochrome c binding site needs to be highly acidic to promote binding of cytochrome c which, if not highly basic overall, at least has a conserved basic patch around the heme cleft. Spinach ***b*$_6$*f***

complex, which interacts with acidic plastocyanin, has a very low affinity for anion exchange resins (unpublished observation, EAB).

A size exclusion chromatography step (Sepharose CL-6B) is included to isolate a single aggregation state, the physiological dimer. The first fractions of the ***bc*$_1$** peak from this column contain significant amounts of cytochrome oxidase. Since most of the cytochrome oxidase elutes from the anion exchange column at much lower ionic strength, and since dimeric oxidase would be smaller than ***bc*$_1$** and thus would elute after the peak, it is assumed that this oxidase is associated with the ***bc*$_1$** complex. In this case, however, there is no evidence for a functional association. It may be that, with this particular ratio of detergent to protein in the extraction, some of the protein-lipid-detergent mixed micelles contain more than one protein complex; and that these higher aggregation states are stable at the low detergent concentrations used in the rest of the preparation. In any case, the sizing column is important to obtain pure material of monodisperse aggregation state. For this column we use a buffer containing 20 mM K-MOPS pH 7.2, 100 mM NaCl, 0.5 mM EDTA (as preservative) and 0.1 g/l dodecyl maltoside. This buffer, subsequently referred to as "column buffer," is a good one for storing or manipulating the protein, which is soluble to nearly 1 mM (240 g/l) in it.

For ***bc*$_1$** complexes of organisms amenable to genetic engineering, rapid purification using His-tag technologies provides another method.[27,28] To the authors' knowledge this has yet to be implemented for a eukaryotic organism.

12.3. Early *bc*$_1$ and *b*$_6$*f* Crystallization Studies

The first three-dimensional structure information available for cytochrome reductase came from electron microscopic studies of 2-D crystals of the enzyme from *Neurospora* mitochondria by Weiss, Leonard and coworkers.[29,30] The two-dimensional crystals were in the space group $P2_12_12$, with rows of dimers with alternating up-down orientation spaced by 111 Å along the diagonals of the unit cell and rows of same-orientation dimers spaced by 137 Å along the *a* axis and 174 Å along the *b* axis.

A later, higher-resolution, electron diffraction investigation of the complex[31] used the purified bovine enzyme reconstituted into tubular helical crystals. The complex was

inserted in one orientation, with the matrix side inside the tubes, with approximately 11 dimers per turn of the helix. Diffraction datasets were calculated from images, merged, and used to calculate the 3-D structure by helical reconstruction. The intrinsic resolution was estimated at 16 Å.

Mosser, Breyton, and co-workers studied thin three-dimensional crystals of the ***b_6f*** complex by electron crystallography and arrived at projection maps based on negative-stained[32] and ice-embedded[33] samples, in the presence and absence of inhibitors.[34] Since the focus of this report is on crystallization in three dimensions for X-ray diffraction, the methods will not be discussed, however they are available in the references.

Another approach for obtaining structural information about membrane proteins is to prepare, by proteolysis or genetic engineering, soluble domains that can be crystallized as soluble proteins to obtain a structure for part of the enzyme. Thus Martinez and co-workers[35,36] crystallized the extrinsic domain of cytochrome ***f*** from turnip and solved the structure by MAD phasing. Similar constructs have been crystallized from *Chlamydomonas*[37,38] and the cyanobacterium *Phormidium laminosum.*[39] Link and co-workers[40] crystallized the extrinsic domain of the bovine Rieske ISP. The structure (entry 1RIE) was obtained by Iwata *et al.*[41] at a resolution of 1.5 Å. Later the structure of a similar construct from the chloroplast ISP (entry 1RFS) was solved.[42] Again the methods will not be described, as they do not directly pertain to crystallization of membrane proteins.

12.4. Three-Dimensional Crystals of Mitochondrial Cytochrome bc_1

Aside from some early reports of microcrystals,[43,44] the first 3-D crystallization of a bc complex was reported by Gros *et al.*[45] These authors reported needle crystals of cytochrome reductase as long as 0.7 mm. They used cytochrome reductase purified using ammonium sulfate and cholate (the specific procedure was not given). The cholate was exchanged for octyl glucoside or POE detergents by dialysis. The buffer was 100 mM KPi, pH 6.7. With octyl glucoside (1%) or C_9E_7 (0.35%), crystallization occurred at 20-30% PEG 4000 or 6000. With $C_{10}E_7$ (0.35%), crystallization occurred at 20% PEG 6000 or 20-30% saturated ammonium sulfate. The crystals were not sufficiently large to test X-ray

diffraction with conventional X-ray sources, so the cell parameters, space group, and diffraction limit are not known.

12.5. Large Tetragonal Crystals from the Yu Preparation

In 1991 Yu and coworkers in Stillwater Oklahoma published a procedure for growing crystals[46] from the bovine enzyme purified by splitting succinate:cytochrome ***c*** reductase as described.[10] The final pellet from NH_4OAc precipitation was redissolved in 50 mM Tris-HCl pH 8.0, 1 mM EDTA, and 0.66 M sucrose, with no detergent other than the cholate carried over in the pellet. This was mixed with an equal volume of precipitating solution yielding initial concentrations of 25 mM Tris-HCl, 0.33M sucrose, 0.25 M NaCl, 6% PEG 4000, 0.04% decanoyl N-methylglucamide (DMG), 1.8% heptanetriol, and 0.05% NaN_3 and subjected to vapor diffusion against a reservoir of higher osmolarity. Crystals grew in 2-4 weeks. Square or octagonal plates were formed, with crystals as large as 4 × 2 × 1 mm. In later experiments[47] the Tris buffer was replaced with 50 mM MES buffer pH 7.0. These crystals diffracted to 4.5 Å, but a note was added in proof that crystals diffracting to 3.5 Å had been obtained, and by the time the structure was reported[48-50] the resolution had improved to 2.7 Å. The space group was $I4_122$, with cell dimensions 153.5 × 153.5 × 597.7 Å (final submitted structure 1QCR).

12.6. Monoclinic, Tetragonal, and Hexagonal Crystals from the Rieske Preparation

Matsubara's group[51] in Osaka published crystallization of the ***bc₁*** complex soon after the report from Yu's lab. They purified the protein from beef hearts using Rieske's method[8] with a final step of polyethylene glycol precipitation from histidine-sucrose buffer with sucrose monolaurate detergent. The crystals were monoclinic with unit cell dimensions 196 × 179 × 253 Å and β=97°. The crystals diffracted synchrotron radiation (λ=1.04Å) to a resolution of 7.5 Å at room temperature. A Matthew's coefficient of 4.4 $Å^3$/Da was calculated assuming two dimers in the asymmetric unit. More details about factors affecting the crystallization were published in a paper[52] with the Yoshikawa's group in Himeji, discussing crystallization of large membrane protein complexes such as the ***bc₁*** complex and cytochrome oxidase. The role of detergent as a precipitant was

demonstrated; addition of excess detergent to a clear solution of the protein resulted in turbidity which was shown to result from microcrystals. While these results were obtained with polyoxyethylene detergents, we have observed a similar effect with octyl glucoside in mitochondrial and bacterial ***bc*$_1$** complexes.

Two new crystal forms were reported in 1994[53] and in the 1992 and 1993 activity reports from the "Photon Factory", Tsukuba Japan. The protein, purified in cholate detergent, was exchanged into sucrose monolaurate by precipitation with PEG. The buffer was changed to KPi by dialysis. Crystallization was by the batch method in a phosphate/SML buffer containing 40-80 g/l protein, 30-50 mM KPi, 0.66 M sucrose and 5 g/l sucrose monolaurate; after adding sufficient PEG 4000 (5-9%) to induce a slight turbidity. This yielded hexagonal column crystals having a hexagonal unit cell with a long c axis (720 Å) and a 6-fold screw axis along c. Assuming a dimer in the asymmetric unit gave a Matthew's coefficient of 3.9 Å^3/Da. Inclusion of Zn^{++} ions at 1 to 4 mM improved the reproducibility of crystallization, without significantly affecting cell parameters. This crystal form has been obtained by other groups (described below) and in the Uppsala lab provided the structure deposited as 3BGY.

The other new crystal form was obtained under similar conditions, but using protein first batch crystallized by ultrafiltration in Tris-HCL and sucrose monolaurate without PEG (giving the original monoclinic form). The microcrystalline material was harvested and it was found that some co-purifying polypeptides had been removed. The crystals were redissolved and dialyzed into the phosphate/SML buffer described above in this section; the resulting solution required higher PEG concentration (8-15%) to reach turbidity. This yielded crystals in a tetragonal space group with the longest axis c=445 Å, $P4_1$ or $P4_3$ symmetry, and Vm= 4.4 Å^3/Da assuming two dimers in the asymmetric unit. The hexagonal crystals grown in the presence of Zn^{++} and the tetragonal crystals both diffracted X-rays to about 6.5 Å.

A later report from Osaka and Himeji[54] described growing the hexagonal column crystals in 40 mM KPi pH 6.5, 660 mM sucrose, and 5 g/l sucrose monocaprate by addition of PEG 4000 to a final concentration of 1.5-2.1% (protein concentration 90 g/l). This markedly lower PEG concentration is consistent with the lower pH and the fact that

solubility decreases with decreasing pH. A precrystallization was performed by concentrating the protein in the same buffer, with no PEG at all. In this case no subunits were removed by the precrystallization. The cell edges of the final diffraction quality crystals were a=b=128.5, c=715.7; and the diffraction limit at 276 K was 2.8 Å.

12.7. Crystallization of the bc_1 Complex from Various Vertebrate Organisms in Berkeley

Berry *et al.*[55] published crystallization of the bovine bc_1 complex, purified by the dodecyl maltoside/ion exchange method, in 1992. Hexagonal bipyramidal crystals were grown in 50 mM KPi pH 6.8, 10 g/l OG, 7.5-12% PEG, and 15-25% glycerol. The crystals diffracted synchrotron radiation to 4.7 Å with film as detector, and belonged to the space group $P6_122$ or $P6_522$.

In 1995, we[56] reported two new crystal forms of the bovine enzyme: hexagonal rod (or column) crystals with a c axis of 752 Å, and another form, which was erroneously assigned an orthorhombic space group, but after further study proved to be monoclinic with space group $P2_1$.

The protein in 50 mM KPi pH 7.5, 300 ml/l glycerol, and 0.5 mM EDTA was supplemented with 1/15 volume of 1 M octyl glucoside and mixed with an equal volume of a precipitant containing 100 mM KPi pH 6.7, 3 mM NaN_3, and 10% PEG. Droplets of the protein-precipitant mixture were equilibrated against a reservoir buffer consisting of 100 mM KPi pH 6.7, 3 mM NaN_3, and PEG-4000 at concentrations of 12, 30, or 35%. Hexagonal rods formed initially under all conditions.

With the lower osmolarity reservoir, the droplets were actually diluted by vapor diffusion, and hexagonal bipyramids began to grow. In some cases the rods were replaced by bipyramids, in others they coexisted. With the highest reservoir osmolarity the hexagonal rods were replaced by the new monoclinic crystals. These crystals diffracted to about 3.8 Å with synchrotron radiation and imaging-plate detectors, comparable to the diffraction limit of the original ($P6_522$) form using the same data collection methodology.

In 1998, these authors reported a structure determination[57] based on four different crystal forms. Two of these were the original hexagonal bipyramids (now shown to be of the $P6_522$ enantiomorph) and the monoclinic crystals from the bovine enzyme. The two

new forms were (1) a hexagonal bipyramid form from the rabbit enzyme, with cell dimensions similar to the beef hexagonal bipyramids, and (2) an orthorhombic crystal of the chicken enzyme. The monoclinic beef crystals and the orthorhombic chicken crystals both had 2-fold noncrystallographic symmetry.

Growth conditions for the rabbit crystals were not published but will be outlined further below. The chicken ***bc*$_1$** complex was isolated by the same procedure used for the bovine. After the final size exclusion chromatography the protein was concentrated to around 200 μM by ultrafiltration through an Amicon YM-100 membrane, precrystallized by mixing with 100 mM KMES pH 6.5 and 10% PEG-4000. The precipitated material was redissolved in column buffer (20 mM K-MOPS pH 7.2, 100 mM NaCl, 0.5 mM EDTA, and 0.1 g/l dodecyl maltoside) containing 20 g/l n-octyl-β-**D**-glucopyranoside. Droplets were set up with equal volumes of the protein solution and a precipitant containing 20 mM KMES pH 6.7, 75 mM NaCl, 10% glycerol, and 6% PEG 4000, then equilibrated by vapor diffusion against 30% glycerol. The chicken crystals frequently diffracted to around 3.0 Å. The structures submitted to the PDB by these authors in 1998 were refined against data from chicken crystals.

12.8. Improved Beef *bc*$_1$ Crystals from the Jap Group at Berkeley and the Iwata Group in Uppsala

Iwata *et al.*[58] published the structure of the bovine ***bc*$_1$** complex based on crystals of the hexagonal bipyramid ($P6_522$) and hexagonal column ($P6_5$) types. The diffraction limit of both crystal forms was around 3.0 Å, much better than we have seen with these forms. The authors attribute this to "dehydration using a heavy metal compound". They found that addition of the high-CMC detergent hecameg (HM) favored formation of the hexagonal columns; bipyramids were obtained in 0.15 g/l DM (no added detergent above that present from the purification). Initial conditions in the hanging or sitting drops calculated from details in reference[59] were: 30 mM KPi pH 6.5-6.9, 50 mM NaCl, 1.5 mM NaN_3, and 2.5-4% PEG 4000. After submission of these structures the resolution was further improved to 2.5 Å, allowing assignment of a number of water molecules.[59]

12.9. Higher Resolution Crystals of the Fungal bc_1 Complex from MPI-Frankfurt

The use of antibody fragments as an aid in membrane protein crystallization has been described in the article.[60] Crystallization of the Yeast (*Saccharomyces cerevisiae*) cytochrome $\boldsymbol{bc_1}$ in complex with an antibody F_v fragment directed against it was reported in 1998.[61] The structure was reported at 2.3 Å in 2000,[62] PDB entry 1EZV. The $\boldsymbol{bc_1}$ complex was purified by a variation of the dodecylmaltoside/anion exchange method,[62,63] intentionally delipidating the complex and depleting it of the weakly bound smallest subunit in order to obtain a homogeneous preparation. This was mixed with purified F_v fragment from an *E. coli* expression system, and the co-complex was isolated by size exclusion chromatography on a TSKgel G 4000 SW column.

The $\boldsymbol{bc_1}$-Fv complex in 20 mM KPi pH 7.5, 250 mM NaCl, and 0.5 g/l UM was concentrated by ultrafiltration to around 50 g/l, mixed with 1 to 2 volumes of precipitant solution containing 5-6% PEG 4000, 100 mM TrisHCl pH 8.0, and 0.5 g/l UDM, and allowed to equilibrate by the sitting drop vapor diffusion method against the undiluted precipitant solution. Crystals grew in 2-3 weeks. This time was shortened to 1-3 days and crystal size improved using a microseeding technique. The crystals are centered monoclinic (space group C2) with a monomer in the asymmetric unit. Phospholipids were present and identifiable,[64] PDB entry 1KB9. Recently this group has crystallized the yeast enzyme in complex with cytochrome ***c*** as well as the Fv fragment, in a different space group with a dimer in the asymmetric unit and cytochrome ***c*** bound to one of the monomers[65], PDB entry 1KYO).

12.10. Unpublished Observations from the Berkeley Group

Needle crystals of the bovine $\boldsymbol{bc_1}$ complex. The initial crystallization attempts at Berkeley were performed by one of us (LSH) in 1990 using material left over from a project on comparative biochemistry of ***bc*** complexes (EAB) which failed to recruit funding. The first crystallization experiments were designed based on the conditions found by Gross *et al.*,[45] using low pH but relatively high ionic strength. The final step of the purification had been dialysis against 50% glycerol, so the crystallization setup contained, in addition, a high concentration of glycerol. Glycerol and increasing ionic strength both

tend to increase the solubility of the ***bc*$_1$** complex under these conditions, therefore relatively high concentrations of PEG were required to obtain any kind of precipitation. Fortunately one of the first crystallization setups resulted in needle-shaped structures growing out of little spherules. A typical well is shown in Figure 12.2. The needles displayed no flat facets or straight edges and tended often to be branched. Still, they were regular enough to stimulate further enzyme preparations and crystallization trials, especially after Werner Kuhlbrandt, who had recently published the most authoritative review of the time on membrane protein crystallization,[66] looked at the crystals in August 1990 and felt that there was a good chance that they were in fact crystalline.

These needle crystals were tested for X-ray diffraction in 1991 using synchrotron radiation (SSRL BL 7-1) with film as detector. The lattice was hexagonal with a 6-fold screw along the long c axis (~700 Å). By that time the hexagonal bipyramid crystals described below were available, with more promising appearance and diffraction as well as more manageable cell dimensions, so we did not further pursue the needle crystals. The hexagonal rod (column) crystals reported by Kawamoto *et al.*[53] in 1994 and by us in the 1995 paper[56] had cell dimensions close to those of the needle crystals. We assumed they were a more refined form of the needle crystals, although the diffraction was not significantly improved. The diffraction of this crystal form was greatly improved by Murakami *et al.*,[54] apparently by changing from sucrose monolaurate to sucrose monocaprate, and by Iwata *et al.*[58] Interestingly, while the crystals shown by Murakami *et al.* were exquisitely shaped hexagonal rods with parallel faces and perfectly square ends, the equally well-ordered crystals from Uppsala were (judging by the Fig. 4 of the article,[59] p 29) irregular rods looking very much like our first crystals in 1990. One observation we can make from our work with the ***bc*$_1$** crystals is that there seems to be no correlation whatsoever of the visual quality of a crystal (i.e., the smoothness of its facets and sharpness of its edges), with the internal order measured by the diffraction limit. This should seem reasonable enough, as a crystal in which the individual molecules are miss-positioned by 20 - 30 Å would still form optically flat facets as long as one layer fills up before the next starts, however diffraction would be limited to a few spots around the beamstop. Conversely, a very highly ordered crystal could have a rough appearance if

cooperativity of adding to one face is not high enough, or if growth runs into an uneven surface such as the surrounding amorphous precipitate. Thus it is important, in selecting crystals to test for diffraction, to be able to distinguish rough but crystalline masses from amorphous ones, and not to be prejudiced against the ugly crystals. Another intriguing crystal form, which we never characterized, is illustrated in Figure 12.3.

Hexagonal bipyramid crystals ($P6_522$). The first hexagonal bipyramids were grown in 50 mM KPi pH 6.7, 25 mM K-MOPS pH 7.3, 12.5% PEG 4k, 34 mM OG, and 250 ml/l glycerol (initial conditions before vapor diffusion). At first they were obtained infrequently and only after several weeks incubation, often in wells that initially developed needle crystals. Perhaps as a result of the late nucleation, size was limited to around 150 μm. Attempts to grow larger hexagonal bipyramids by the macro-seeding technique led to interesting results. Although the seeded crystals did not grow significantly in new protein/precipitant mixture, seeding seemed to induce a shower of new crystals of hexagonal bipyramidal shape. Perhaps a large number of microcrystals was present in the mother liquor of the old crystals, or became dislodged from the old crystal during seeding, and served as nuclei for formation of new crystals of the same form. This was reminiscent of the procedure of Fitzgerald and Madsen[67] who homogenized crystals to prepare a seed solution which, used at the right dilution, induced reproducible growth of a small number of large crystals. We adopted this approach, seeding each well with a dilute suspension of crystals washed and homogenized in 40% PEG 2000, 100 mM KPi pH 6.7, 3 mM NaN_3, 0.25 g/l $C_{12}E_{10}$, and 5 g/l OG. Crystals appeared with good reproducibility in the first day or two, and if the number of crystals was small and the protein concentration high (0.2-0.4 mM or 50-100 g/l protein) crystals would grow as large as 1 mm.

Conditions for growth. Using the seeding technique, hexagonal bipyramid crystals were obtained with (initial conditions) 12-13% PEG (2000 or 4000), 23-34 mM OG, 67-100 mM enzyme (wider range not tried), and ionic strength 85-115 mM (usually 25 mM K-MOPS, 50 mM KPi), pH 6.7 and 4°C. An undetermined amount of dodecyl maltoside or polyoxyethylene detergent was present from the protein stock solution.

Although these were considered vapor diffusion experiments, it was later realized that the droplets sometimes had higher osmolarity than the reservoir due to presence of

glycerol in the protein solution, so that vapor diffusion would be in the direction of reducing protein and precipitant concentrations. The crystals could also be grown in X-ray capillaries where the surface area to volume ratio was greatly decreased, thus we doubt if vapor diffusion was important. Crystals grew in capillaries to fill the diameter of even the 1.0 mm capillaries, allowing X-ray data collection without the trauma of crystal mounting and yet without putting a lot of mother liquor in the X-ray beam. This did not significantly enhance resolution, however, suggesting that our low (3.8 Å) resolution was not a result of damage during mounting but rather the intrinsic resolution of the crystals grown under these conditions.

It was during this period that we tried greatly increasing the osmolarity of the reservoir to drive vapor diffusion in the direction of concentrating the protein and obtained the monoclinic crystals of reference[56] rather than hexagonal bipyramids. Monoclinic crystals were only obtained in four experiments, therefore we do not understand the conditions required. The hexagonal bipyramids clearly form with no vapor diffusion or even with dilution by vapor diffusion, suggesting they are forming in the salting-in region of the protein's phase diagram.

Hexagonal bipyramid crystals without seeding. During the search for heavy atom derivatives to use for phasing by multiple isomorphous replacement, it was desired to avoid phosphate, which precipitates many of the heavy atom reagents. The Good[68] buffers MES and MOPS were used. The protein in 20 mM MOPS pH 7.2, 100 mM NaCl, and 0.1 g/l DM was mixed with an equal volume of precipitant containing 10-100 mM MES buffer at pH 6.7, NaCl at 0-100 mM, and PEG 4K at 4-5%. OG was added to either the protein or the precipitant to give a final concentration of 10 g/l.

Crystals were obtained over a large part of this range, most frequently at lower NaCl concentrations (0-25 mM). Calculated final pH and ionic strength in the droplet are in the ranges 6.8-7.0 and 65-100 mM. Rather than settling on precise conditions, this range of conditions was used in every experiment. The slight differences in precipitant compositions may serve to compensate for differences in the batches of protein, so that no one precipitant was always the best. With this protocol seeding was no longer necessary, as a reasonable number of crystals would form without seeding. It remains to be

determined whether seeding would extend the range over which crystals can be obtained, perhaps into a region that gives better quality crystals.

Precrystallization. Around this time we introduced a "precrystallization" step into some of the protocols. The idea was to serve as a final step of purification, in which those molecules with a good propensity to crystallize are selectively precipitated, while deranged molecules that do not fit into the lattice properly are left behind with other contaminants in solution. The initial precrystallization experiments were carried out in 9-well glass trays, adding to the protein (in column buffer) an equal volume of 100 mM KMES pH 6.7, 5% PEG, and 3 mM NaN_3. After about one day the crystals were harvested by centrifugation, redissolved in the column buffer, and used for crystallization. Later the PEG concentration in the "precrystallization" mixture was increased to 10%, so that precipitation was complete in a few minutes. It was not ascertained whether the precipitate was crystalline or amorphous. Thus this served more as a concentration and buffer/detergent exchange step than a real precrystallization.

Rabbit cytochrome *bc*$_1$ crystals. The rabbit ***bc*$_1$** complex was purified by the same procedure as the beef, with detergent:protein ratio of 1:1 in the extraction. After the gel filtration column, the protein was concentrated by ultrafiltration, precipitated by precrystallization as above, and dissolved in column buffer (20 mM KMOPS pH 7.2, 100 mM NaCl, and 0.5 mM EDTA) with 20 g/l OG. Crystals could be obtained under the phosphate-free conditions for the beef enzyme. For example, the enormous crystal of Fig. 12.4 was obtained by mixing protein with an equal volume of precipitant to give 25 mM MES 6.7, 10 mM MOPS pH 7.2, 10 g/l OG, 2.5% PEG 4000, and 1.5 mM NaN_3, then diluting the 40 μl drop with 8 μl of water. Apparently diffraction from this crystal was never tested, probably because no one wanted to try coaxing a 4 mM crystal into the shoulder of an X-ray capillary.

Diffraction was tested on two rabbit crystals grown in Tris-Acetate rather than MES buffer. For these crystals, portions of the redissolved rabbit ***bc*$_1$** were mixed with equal volumes of precipitant containing 5% PEG 4000, 1.5 mM EDTA, and either 75 mM Tris-acetate pH 7.0 plus 25 mM NaCl or 100 mM Tris Acetate plus 10 mM NaCl. Single, large (~ 1 mm) crystals grew in two of the wells; some other wells had several smaller crystals.

Data was collected from the two large crystals at ~0 °C after mounting in capillaries. They diffracted to 3.5 Å, a little better than obtained for any of the beef hexagonal bipyramids at that time.

Since Tris-acetate is not much of a buffer at this pH, it might be considered more of a salt or additive in this procedure, and MOPS from the protein solution the effective buffer. The final pH is thus higher (the exact pH is uncertain because of unknown carryover of MES from the precrystallization) than in the experiments described above where KPi or MES was used in the precipitant to lower the pH.

Orthorhombic Chicken *bc*$_1$ crystals. The first chicken crystals were actually observed in a precrystallization step: ***bc*$_1$** complex from the gel filtration column was concentrated by ultrafiltration and mixed with an equal volume of precipitant containing 100 mM KMES 6.7, 10% PEG 4000, and 3 mM NaN_3. Precipitation was not as rapid as with the beef enzyme, and the sample was left to incubate overnight. The granular appearance of the precipitate prompted a microscopic examination which revealed it to consist of small rectangular prisms. The crystals were harvested by centrifugation and redissolved in column buffer with and without 0.1 g/l DM, but no crystals were obtained from this material.

In the next experiment the precrystallization was accelerated by adding a few drops of 50% PEG 4000, and the precipitate was harvested after 20 minutes. The pellet was dissolved in column buffer without detergent, OG was added to 20 g/l, and portions were mixed with equal volumes of precipitants containing 10-100 mM MES buffer at pH 6.7, 0-100 mM NaCl, 5% PEG 4000, and 3 mM NaN_3. Crystals were obtained in many of these conditions. After that experience the time in the precrystallization buffer was limited to an hour or less for the chicken enzyme.

Figure 12.5 illustrates the crystals of chicken cytochrome bc1. These crystals had several advantages over any of the beef crystals we had obtained: they could readily be equilibrated with cryoprotectant and flash-cooled for cryogenic data collection, the longest cell parameter of only ~240 Å facilitated accurate data collection, and the crystals often diffracted as high as 3.0 Å. This limit was not very sensitive to intensity of the X-ray source, being about the same with a rotating anode generator and at various synchrotron

sources. The asymmetric unit contained a dimer, allowing the use of noncrystallographic restraints during refinement. This was probably crucial to the success of the refinement, given the low resolution and thus poor ratio of data to parameters. Finally the iron-sulfur protein subunit is not involved in any crystal contacts when present in the "cytochrome b" position. Only one monomer makes a contact in the "released" position, and that is a tenuous one which apprently does not affect the equilibrium position of this subunit. This makes this crystal form ideal for investigating movement of the iron-sulfur protein in response to inhibitors, redox state, or other factors.

12.11. Conclusions

Table 12.1 provides a survey of a few of the most important parameters in the crystallization protocols described here. All crystals were grown within one pH unit of neutrality, and with the exception of Yu's crystal at relatively low ionic strength. There is some correlation between the amount of PEG used and the pH (lower pH makes the protein less soluble requiring less PEG) and the presence of glycerol (glycerol increases solubility and so requires more PEG).

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Table 12.1.- ionic strength, pH, and [PEG] from various crystallization conditions.

ref[a]	form[b]	pH	μ[c], mM	PEG	detergent	buffer	other	label[d]
(46)	1 ($I4_122$)	8.0	265	6%	0.4 g/l DMG	TrisCl	NaCl	a
(47)	1	7.0	265	6%	0.4 g/l DMG	MES	NaCl	b
(53)	6 ($P6_5$)	8.0	86-144	5-9%	5 g/l SML,	KPi	sucrose	c
(53)	2 ($P4_{1(3)}$)	8.0	86-144	8-15%	5 g/l SML	KPi	sucrose	c
(54)	6	6.5	67	1.5-2.1%	5 g/l SMC	KPi	sucrose	d
(55)	5 ($P6_522$)	6.8	200	7.5-12.5%	10 g/l OG	KPi	glycerol	e
(56)	4 ($P2_1$)	6.9	161	5%	9 g/l OG	KPi	glycerol	f
(non-phosphate)	5	6.8-7.0	65-96	2-2.5%	10 g/l OG	MES/MOPS	NaCl	g
(rabbit)	5	7.2	100-110	2.5%	10 g/l OG	Tris/OAc	NaCl	h
(57) (chicken)	7 ($P2_12_12_1$)	7.03	102	3%	10 g/l OG	MES/MOPS	NaCl	j
(59)	5	6.3-6.9	92-107	2.5-4%	DM	KPi	NaCl	k
(59)	6	6.3-6.9	92-107	2.5-4%	DM(+HM)	KPi	NaCl	k
(62)	(yeast-Fv)	7.9-7.95	139-177	2.5-3%	0.5 g/l UM	TrisCl/KPi	NaCl	m

a- If no reference number, refers to unpublished results discussed here.

b- Crystal form as classified in Table 1, reference.[1] Crystals with the same space group and similar cell dimensions were assumed to belong to the same crystal form.

c- Approximate ionic strength ($\mu = \frac{1}{2}\Sigma C_i Z_i^2$, where C is the concentration and Z the net charge of each ionic species; in mM units).

d- The letters in the last column refer to pH/ionic strength points on Fig. 12.6.

Abbreviations used: ***bc*$_1$**: cytochrome ***bc*$_1$** complex; PEG: polyethyleneglycol. **Detergents:** OG: octyl-β-D-glucopyranoside, DM: dodecyl-β-D-maltopyranoside, UM: undecyl-β-D-maltopyranoside, DMG: decanoyl N-methylglucamide; SML: sucrose monolaurate; SMC: sucrose monocaprate, HM hecameg ([6-O-(N-Heptylcarbamoyl)methyl-α-D-glucopyranoside]), DOC: deoxycholate. **Buffers:** MES: morpholinoethanesulfonate, MOPS: 3-N-morpholinopropanesulfonate; tris-(hydroxymethyl)aminomethane, Pi: inorganic phosphate, KPi: potassium phosphate.

Figure Legends

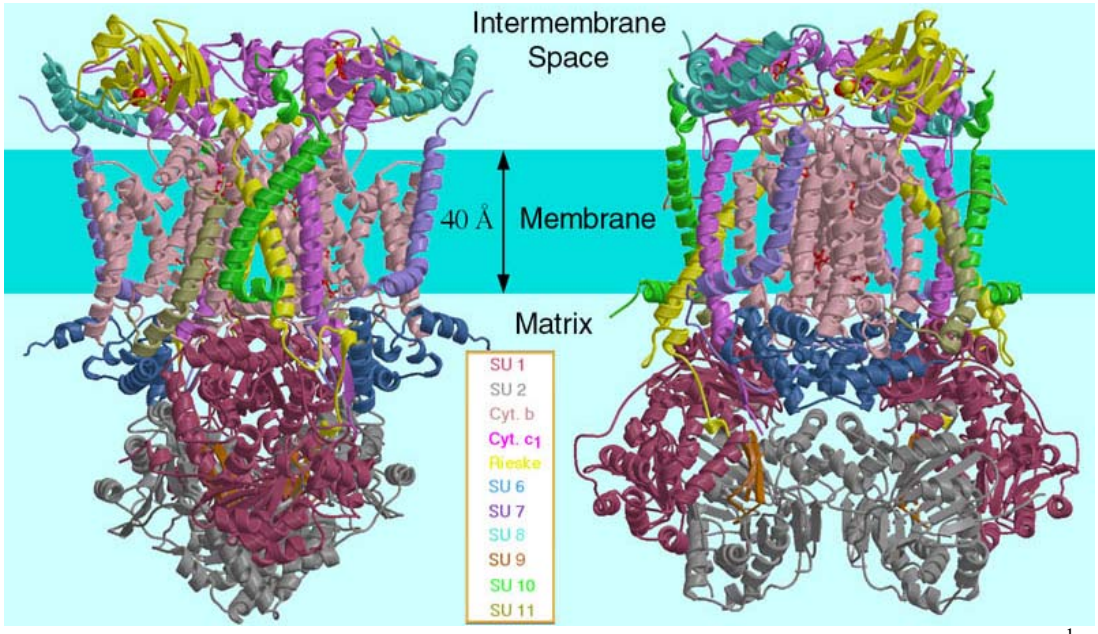

Figure 12.1. Structure of the mitochondrial bc1 complex. This figure (from ref.)[1] is made from a composite of structures 1QCR, 1BCC, and 1BE3. The gray band represents the presumed membrane position. The 11 subunits are color coded as indicated in the key. Two dimers are shown, related by a rotation of 90° within the plane of the membrane (i.e., about the dimer axis). It can be seen that considerable protein extends into the aqueous phase on either side of the membrane.

Figure 12.2. Needle crystals with pinheads, obtained from one of the first crystallization attempts of our group. As described in the text they were grown from bovine ***bc*$_1$** complex in phosphate buffer with glycerol and PEG 4000. The unit cell is hexagonal with a 6-fold screw along the c axis, and the cell edge is about 700 Å along c. It is likely that these belong to the same space group as the hexagonal rod or column crystals described later[53,56] and those used for the structure deposited as 1BGY.[58]

Figure 12.3. Herringbone crystal "tree" of beef *bc*$_1$. Diffraction was not tested.

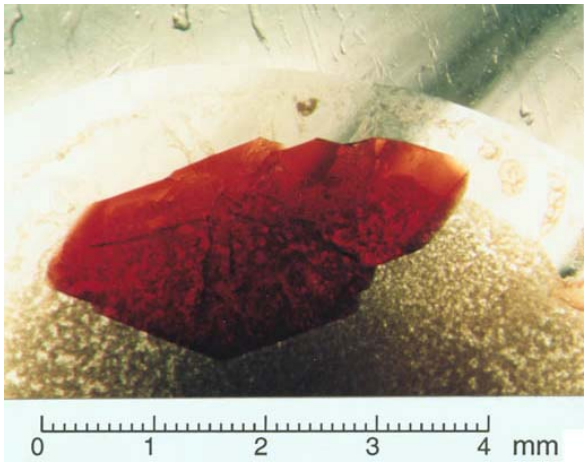

Figure 12.4. Giant crystal of rabbit *bc*$_1$ complex growing in a 9-well depression plate. The crystal was grown in one of the standard phosphate-free crystallization conditions (see text), but the drop was diluted with distilled water just after mixing protein and precipitant.

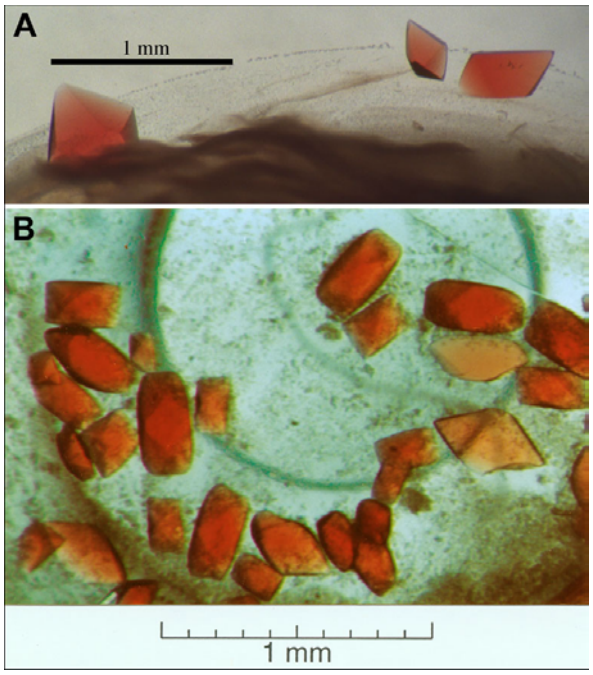

Figure 12.5. Orthorhombic crystals of chicken *bc*$_1$. A. Unusually large and sharp-looking crystals in their native setting. These crystals are visible in a clear zone around the edge of the well; the center of the well is obscured by amorphous precipitate. B. More typical crystals, after rinsing to remove most of the precipitate.

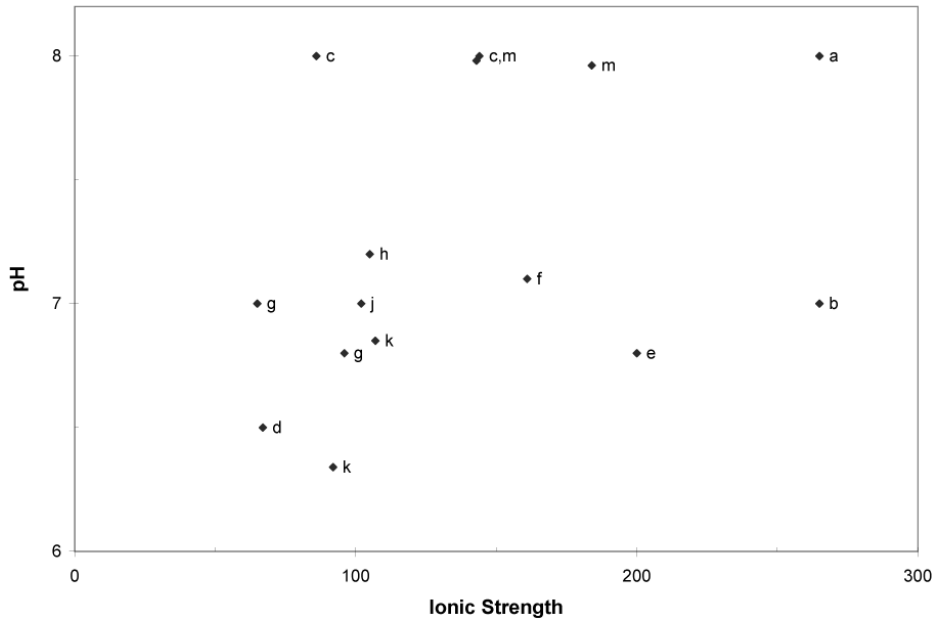

Figure 12.6. pH and ionic strength conditions used in the crystallization mixtures reported here, taken from Table 12.1. A relatively narrow range of pH and ionic strength has yielded crystals. Within that range the PEG concentration has to be adjusted to compensate for effects of pH, ionic strength, and glycerol on the solubility of the protein. The letters labeling the points refer to the corresponding line in Table 12.1.